Potential of Concentrated Animal Feed Operations (CAFOs) To Contribute Estrogens to the Environment

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Concentrated Animal Feed Operations (CAFOs)

- In the United States, an estimated 376,000 animal feed operations confine animals, generating approximately 128 billion pounds of manure each year
- A facility is an animal feed operation (AFO) if animals are stabled/confined, or fed/maintained, for 45 days or more within any 12-month period, and the facility does not produce any crops, vegetation, or forage growth
- Concentrated animal feed operations (CAFOs) are the largest of these and are regulated under the Clean Water Act. CAFOs are generally considered to be operations with more than 1000 animal units (AU)

Manure Available for Land Application, 1997*

Sector	Total Manure (billion pounds)	Percentage Share by > 1000 AU Operations
Cattle	32.9	83%
Dairy	45.5	23%
Swine	16.3	55%
Poultry	33.5	49%

*Office of Wastewater Management (Office of Water/USEPA) Website: (http://cfpub1.epa.gov/npdes/home/cfm)

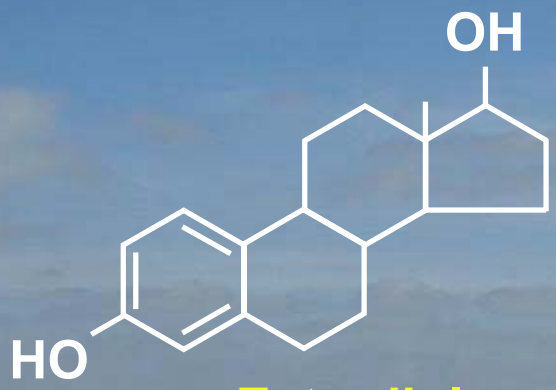
Environmental Estrogens

- Refer to a wide range of anthropogenic or naturally occurring compounds that elicit estrogenic responses by mimicking endogenous estradiol

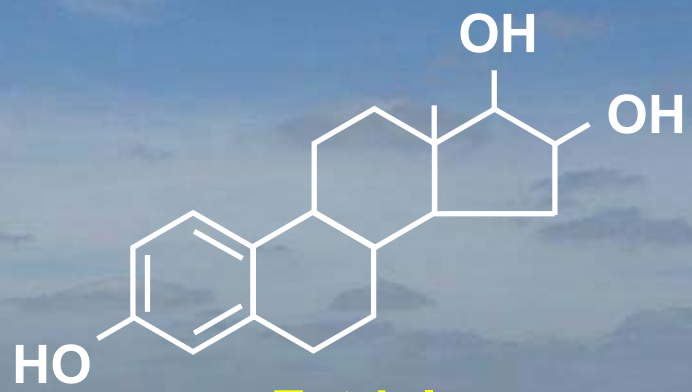
- Natural Estrogens:
  - 17β-Estradiol ("Estradiol"), Estriol, Estrone, Equilin, Equilenin, Genistein

- Synthetic Estrogens:
  - Ethinyl estradiol, Mestranol, Diethylstilbestrol

- Other Compounds
  - *o, p'* – DDT, Nonylphenol, Bisphenol A

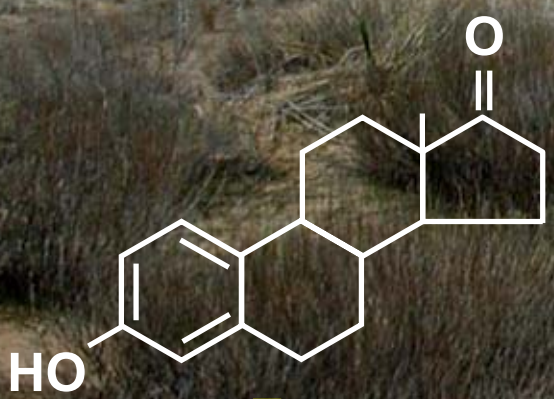


Estradiol

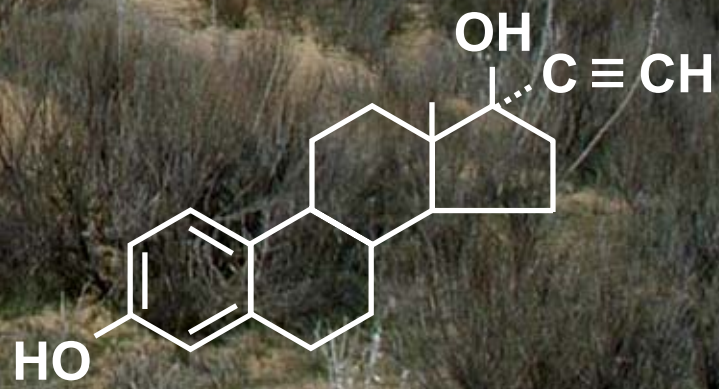


Estriol

Estrogen “Core”



Estrone



Ethinyl Estradiol

Comparison of Estrogenic Activity in Terms of EC50 Measured by Yeast Estrogen Screen*

Substance	Relative Ratio of Estrogenic Activity
Natural Estrogens	
Estradiol	1.0
Estrone	0.21
Estriol	0.0013
17β-Estradiol-3-Sulfate	0.000053
Phytoestrogens	
Genistein	0.00011
Other Compounds	
Nonylphenol	0.001
Bisphenol A	0.00027

*Matsui et al, 2000

Comparison of Estrogenic Activity in Terms of Plasma Vitellogenin Induction*

Substance	Minimum Aqueous Concentration Required for Vitellogenin Induction
Natural Estrogens	
Estrone	100 ng/L
Estradiol	10 ng/L
Synthetic Estrogens	
Ethinyl Estradiol	2 ng/L

*Arcand-Hoy et al, 1998

Use and Expected Environmental Impact of Estrogenic Pharmaceuticals Prescribed and Sold in the U.S.*

Pharmaceutical Estrogen Product	Calculated Estrogen Use	Expected Introduction Concentrations to the Aquatic Environment
Human Use		
Oral Contraceptives (ethinyl estradiol, mestranol)	88 kg/yr	2.2 ng/L
Hormone Replacement Therapy (conjugated estrogens)	1700 kg/yr	42 ng/L
Animal Use (Cattle Only)		
Growth-Enhancement (estradiol)	580 kg/yr	14 ng/L

*Arcand-Hoy et al, 1998

CAFO Contributions of Estrogens - Cattle

- Estimated that at least 90% of feedlot cattle slaughtered in 1995 were administered growth-enhancing hormones
- Growth Hormones
  - Estrogens (estradiol, estradiol benzoate)
  - Androgens (trenbolone acetate, testosterone propionate)
  - Progestins (progesterone)
- For cattle, the estradiol concentration in the urine averages 13 ng/L
- Cattle subjected to growth hormones generate urine with estradiol concentrations five- to sixfold greater

CAFO Contributions of Estrogens - Poultry

- No growth hormones added: natural production of estrogens and testosterone
- In 1998, the U.S. poultry industry produced almost eight billion broilers with a total production of almost 12 billion kg litter
- Average estimated hormone concentrations per kg dry weight litter:
  - 14 μg estrogens (estradiol, estrone) in male broilers
  - 65 μg estrogens (estradiol, estrone) in female broilers
  - 133 μg testosterone in male and female broilers
- Estimated estrogen production: 160,000 – 760,000 kg/year
- Field study shows sizeable edge-of-field losses of estradiol (20-2530 ng/L) and testosterone (10-1830 ng/L) in runoff from litter-amended grasslands (Finlay-Moore et al, 2000)

CAFO Contributions of Estrogens - Swine

- No growth hormones added: natural production of estrogens and testosterone

- Estrogen production in swine:

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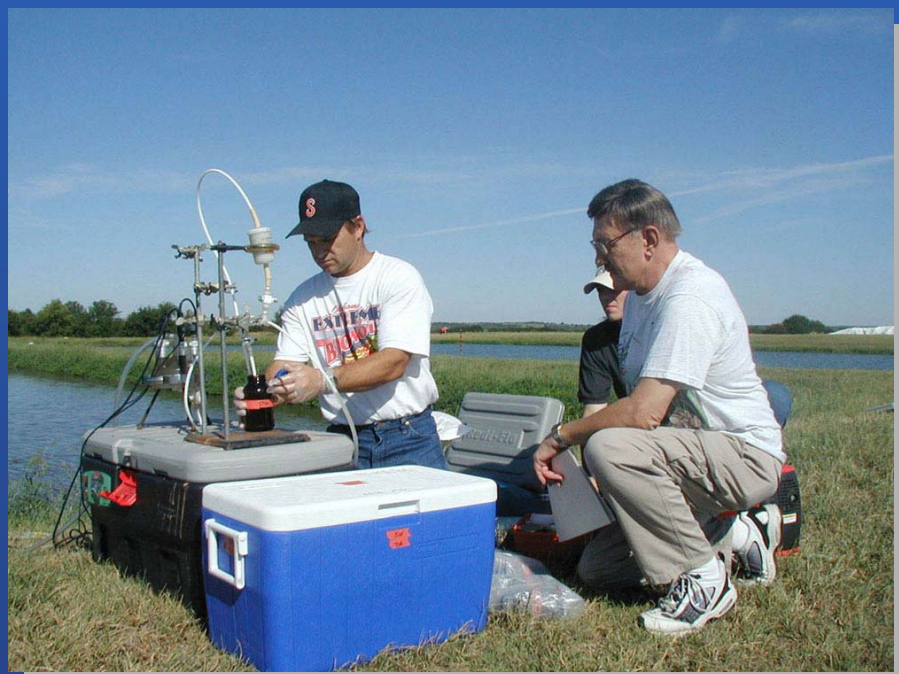
Evaluation of CAFO Lagoon Effluents for EDC Activity using Bioassays



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OSU CAFO EDC Study - Objective

The objective of this study is to evaluate lagoon samples from swine, beef, and dairy CAFOs for possible EDC activity, using a variety of tests based upon the African Clawed Frog (*Xenopus laevis*)

OSU Swine Lagoon

OSU Beef Lagoon

OSU Dairy Lagoon

OSU CAFO EDC Study – Test Description

FETAX
(Frog Embryo Teratogenesis Assay – *Xenopus*)

Adult Male Frog Exposure -
Vitellogenin Expression
and Estradiol/Testosterone Changes

XTRA
(*Xenopus* Tail Resorption Assay)

OSU CAFO EDC Study – Test Description

FETAX - Frog Embryo Teratogenesis Assay - *Xenopus*

- **Standardized Protocol (ASTM Standard Guide E 1439-91)**
- **96-hr assay that uses early stage *Xenopus* embryos (Stages 8-11, approximately 8-12 hours old)**
- **Measures growth, mortality, and malformation for detection of potential developmentally toxic compounds and mixtures**
- **Not used for detection of EDC activity per se; needed for screening samples for potential toxicity to determine allowable concentrations**

OSU CAFO EDC Study – Test Description

XTRA – *Xenopus* Tail Resorption Assay

- **Tail resorption controlled by thyroxin – measures disruption of thyroid gland**
- **Uses Stage 56/57 (approximately 38/40-day old) *Xenopus* larvae**
- **Four replicate tanks, 10 larvae per tank, water changed twice per week**
- **Larvae photographed every other day; tail length measured by SigmaScan software**
- **Test duration 15-20+ days (until metamorphosis is complete)**

OSU CAFO EDC Study – Test Description

Adult Male Frog Exposure – Vitellogenin Expression and Estradiol/Testosterone Changes

- **Vitellogenin Analysis – Exposure of male oviparous vertebrates to natural and synthetic estrogens can induce synthesis of the phospholipoglycoprotein yolk precursor vitellogenin**
- **Estradiol/Testosterone Analyses – Indicator of alterations in reproductive endocrine homeostasis**

Exposure: Four groups of 5 adult male *Xenopus* exposed for 21 days

- **Untreated Controls – reared in charcoal-filtered water**
- **Positive Plasma Controls – reared in charcoal-filtered water; intraperitoneal injection of 1 mg/kg ethinylestradiol on Days 1, 3, and 6**
- **Positive Aqueous Controls – reared in charcoal-filtered water with 1 mg/L ethinylestradiol**
- **Test Group – reared in CAFO lagoon effluent**

Analyses

- **After exposure, plasma prepared and assayed for vitellogenin using Western immunoblotting and enzyme-linked immunoassay (ELISA), and assayed for estradiol and testosterone using enzyme-linked immunoassay (ELISA)**

OSU CAFO EDC Study – Conclusions

Conclusions are Preliminary – Analyses Pending

- Although the swine effluent lagoon is quite toxic, none of the lagoons have exhibited significant EDC activity, at least based on these bioassays

- EDC activity may be truly insignificant in these CAFO lagoon effluents

- However:
  - These lagoons may not be truly representative of large-scale commercial operations
  - EDC effects on steroid hormone homeostaesis may be more pronounced under long-term exposure

OSU CAFO EDC Study – Poster Presentation

22nd Annual SETAC Meeting, November 11-15, Baltimore, MD

- ***Lagoon Water from Confined Animal Feed Operations and Amphibian Development***
  Dumont, J.N.*, Oklahoma State University, Stillwater, OK
  Hutchins, S.R., U.S. EPA (NRMRL/SPRD), Ada, OK

- ***Endocrine Modulating Effects of Lagoon Water from Confined Animal Feed Operations on Amphibians***
  Weber, L.P.*, Dumont, J.N., and Janz, D.M., OSU, Stillwater, OK
  Selcer, K.W., Duquesne University, Pittsburgh, PA
  Hutchins, S.R., U.S. EPA (NRMRL/SPRD), Ada, OK

Analysis of Environmental Estrogens in Swine Wastewater, using ELISA, LC/MS/MS, and GC/MS

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HONDA
ADA, OK


RAW
CENTRIFUGED
CENTRIFUGED
GLASS FIBER FILTER
CENTRIFUGED
GW 0.45 FILTER
1000 mL
PYREX
No. 1395

Environmental Estrogen Analysis – Objective

- The objective of this study is to develop a protocol for screening and analyzing swine lagoon effluent and ground water for estrogens at environmental levels (ng/L)
- Screening will be done using enzyme-linked immunoassay (ELISA) specific for estradiol. Positive samples will then be analyzed for individual estrogens
- Individual estrogens will be analyzed by LC/MS/MS
- LC/MS/MS Interferences - switch to GC/MS

Environmental Estrogen Analysis – ELISA Screen

Initial Procedure

- Solid phase extraction of 250-mL sample using 6-mL ENVI-CARB SPE cartridge
- Sequential washing with water, methanol/acetic acid, and methanol
- Elution with methylene chloride/methanol
- Evaporation to dryness; resuspension with water/methanol to 500 µL
- Direct analysis of 20-µL aliquots by ELISA for estradiol
- Estimate of estradiol concentration using external calibration curve

Environmental Estrogen Analysis – ELISA Screen

Preliminary Findings

- **Cross-reactivity: higher concentrations of other estrogens will produce a similar positive response in the estradiol ELISA screen**
  - **Estriol – Response is 1% that of estradiol**
  - **Estrone – Response is 1% that of estradiol**
  - **Ethinyl estradiol – Response is 0.2% that of estradiol**
- **Swine lagoon effluent appears to exert a positive interference, in that estimated estradiol concentrations can be much higher than those confirmed by direct LC/MS/MS analysis**

Environmental Estrogen Analysis – LC/MS/MS Analysis

Initial Procedure (Finnegan TSQ 7000 Mass spectrometer)

- Analyze same SPE extract prepared for ELISA screening
- Micro-liquid chromatography electrospray MS/MS method
- 2-μL sample loop injection onto 5-μL Zorbax C18SB packed capillary column
- Gradient elution with acetonitrile/water
- Addition of ammonium hydroxide to column eluent prior to electrospray source to abstract phenoxy proton from the estrogen
- Quantitate estrogen concentrations using calibration curves with estrone-d_4 as the internal standard

Preliminary Results of Swine Lagoon Estrogen Analysis

(all concentrations in ng/L original water sample)

Sample ID	Type	ELISA	LC/MS/MS Analysis			
		estradiol	estriol	estradiol	ethinyl estradiol	estrone
#1	Lagoon	27.2	5?	3?	0	285
#2	Well	0.1	NA	NA	NA	NA
#33	Lagoon	69.0	0?	0?	20	82
#40	Well	1.3	0?	1?	0	0
#41	Lagoon	21.4	0?	0?	0	10
#51	Well	0.3	0?	1?	0	0
#52	Lagoon	26.6	0?	2?	0	5
#63	Lagoon	6.0	2?	3?	2	10
#64	Well	1.3	0?	1?	0	0
#71	Lagoon	31.2	2?	3?	0	85
#72	Lagoon	81.4	2?	0?	0	246
#73	Lagoon	22.8	6?	9?	5	187
#80	Well	0.3	0?	0?	0	0
#81	Lagoon	84.8	0?	0?	0	34
#84	Lagoon	56.0	0?	0?	0	51

* Not Analyzed

Environmental Estrogen Analysis – LC/MS/MS Analysis

Potential Problems

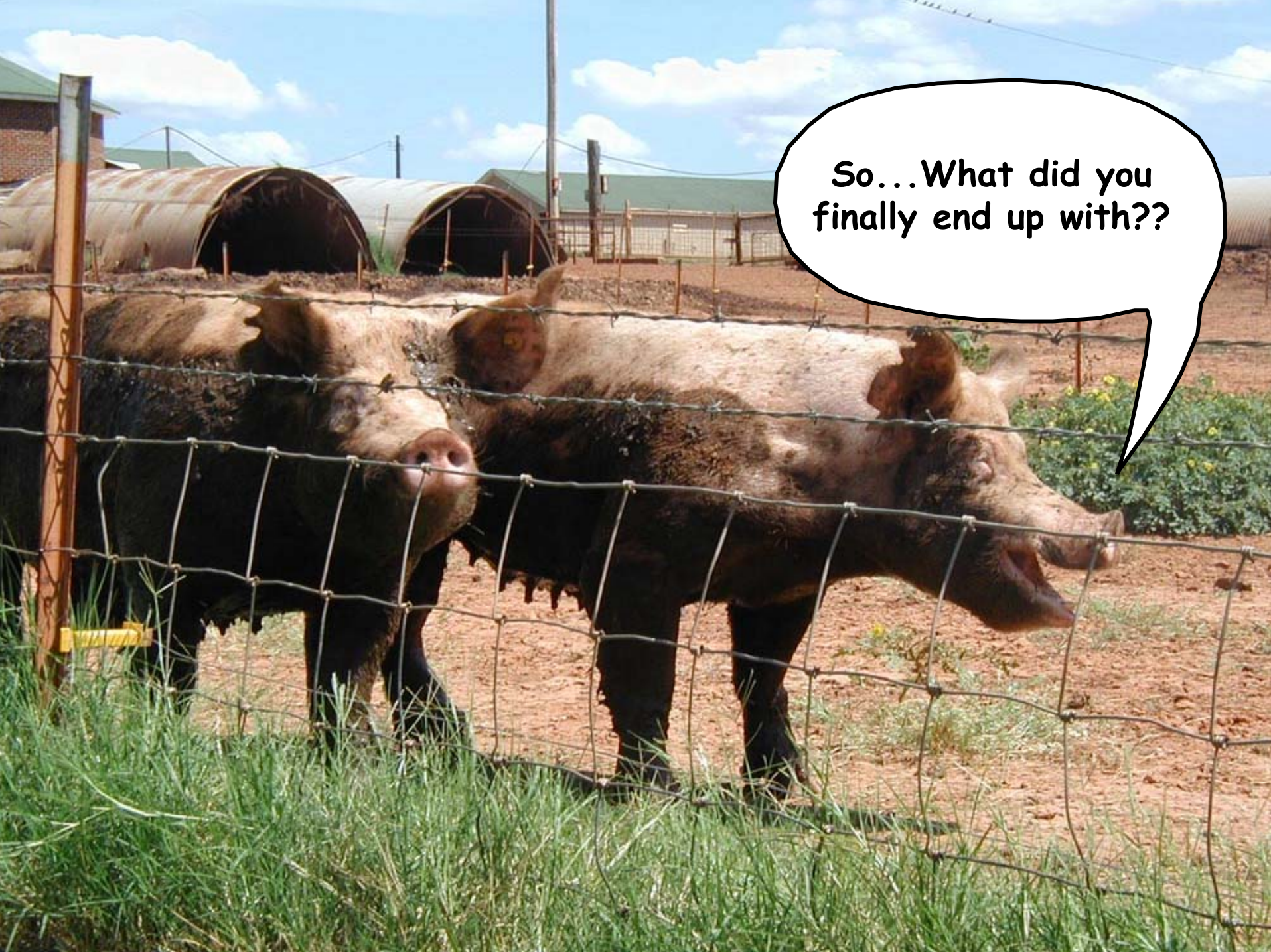
- LC/MS/MS analysis shows numerous organic compounds that elute in early part of chromatograph that may cause positive response with ELISA screen

- Estrogenic response in LC/MS/MS (electrospray) system is suppressed when high organic interferences coelute with compounds of interest (insufficient clean-up)

Possible Solutions

- LC clean-up of complex samples (e.g., swine lagoon effluents) prior to ELISA screen, using gel permeation chromatography and silica gel prep to remove early-eluting interferences

- Evaluate other SPE cartridges and/or alternate analytical techniques


So...What did you finally end up with??

Environmental Estrogen Analysis – SPE Method

Final Procedure

- **Solid phase extraction of 500-mL water sample or 25-mL swine lagoon effluent sample using OASIS HLB SPE cartridge**
- **Sequential washing with water/methanol, water, and methanol/aqueous ammonium hydroxide**
- **Elution with MTBE/methanol**
- **Evaporation to dryness;**
  - **for ELISA screen, resuspend with methanol to 250 *µ*L and dilute 1:2 with water**
  - **for GC/MS analysis, resuspend with acetone to 1000 µL**

Environmental Estrogen Analysis – ELISA Screen

Final Procedure - used with water samples only

- Direct analysis of 20-μL aliquots by ELISA for estradiol
- Estimate of estradiol concentration using external calibration curve
- Concentration factor = 1000; detection limit ~ 0.05 ng/L estradiol in original water sample

Environmental Estrogen Analysis – GC/MS Analysis

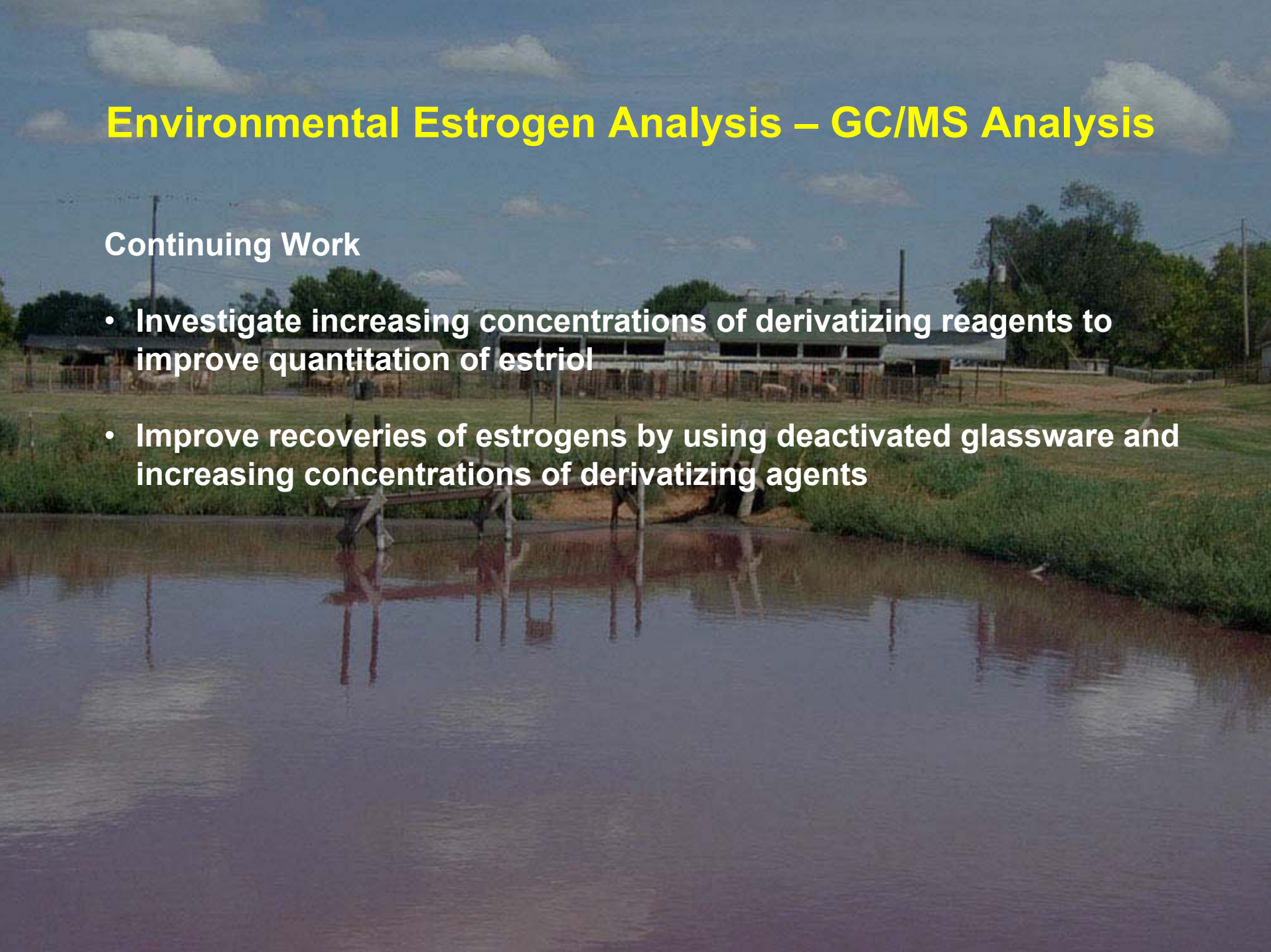
Final Procedure (Finnigan 4600 Mass Spectrometer)

- **Prepare pentafluorobenzyl derivatives of phenolic groups and trimethylsilyl derivatives of hydroxy groups**
- **Analyze the derivatized estrogens by GC/MS using a J&W DB5-MS capillary column and negative ion chemical ionization mass spectrometry**
- **Quantitate estrogen concentrations using internal calibration curves and estrone-d_4, estradiol-d_3 and ethinyl estradiol-d_4 as internal standards**
- **Concentration factor = 25 (lagoon effluent) and 1000 (ground water); detection limit ~20 ng/L estrogen in lagoon effluent and ~0.5 ng/L in ground water**

Recovery of Estrogens Spiked in Distilled Water and in Swine Waste Lagoon Samples

% Recovery	Distilled Water Spiked at 2 ng/L	Distilled Water Spiked at 1 µg/L	Lagoon Effluent Spiked at 1 µg/L (duplicates)	
7-α-Methylestrone (surrogate)	110	85.2	86.2	87.4
Estrone	110	83.2	68.8	58.8
Estradiol	160	84.0	73.6	83.2
Ethinyl estradiol	110	90.8	86.0	88.0
Estriol	210	115	75.4	109

Environmental Estrogen Analysis – GC/MS Analysis



Continuing Work

- Investigate increasing concentrations of derivatizing reagents to improve quantitation of estriol
- Improve recoveries of estrogens by using deactivated glassware and increasing concentrations of derivatizing agents